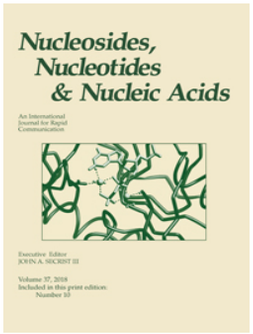

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Synthesis of oligodeoxyribonucleotides containing a tricyclic thio analogue of O^6-methylguanine and their recognition by MGMT and Atl1

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ABSTRACT

Promutagenic O^6-alkylguanine adducts in DNA are repaired in humans by O^6-methylguanine-DNA-methyltransferase (MGMT) in an irreversible reaction. Here we describe the synthesis of a phosphoramidite that allows the preparation of oligodeoxyribonucleotides (ODNs) containing a novel tricyclic thio analogue of O^6-methylguanine in which the third ring bridges the 6-thio group and C7 of a 7-deazapurine. These ODNs are very poor substrates for MGMT and poorly recognised by the alkyltransferase-like protein, Atl1. Examination of the active sites of both MGMT and Atl1 suggest large steric clashes hindering binding of the analogue. Such analogues, if mutagenic, are likely to be highly toxic.

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Tricyclic; O6-methylguanine; oligodeoxyribonucleotide; MGMT; alkyltransferase-like protein

1. Introduction

The exposure of DNA to alkylating agents can result in the formation of toxic, pro-mutagenic O^6-alkylguanine (O^6-alkG) adducts (e.g., Figure 1). Insertion of thymine opposite O^6-alkGs during DNA replication can result in GC->AT transition mutations, that are a common feature of many tumours[1] and In humans the protein O^6-methylguanine-DNA-methyltransferase (MGMT) repairs O^6-methylguanine [O^6-MeG (**1**)] and other O^6-alkGs *via* the irreversible transfer of the alkyl group to Cys145, thereby reforming guanine and inactivating the protein.[2] During this process the

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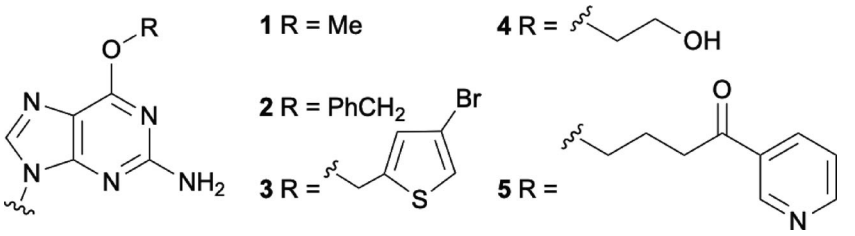


Figure 1. Structures of selected O^6-alkylguanine adducts.

damaged base is flipped from the DNA duplex into the protein's active site.[3] In humans, the repair of O^6-alkGs by MGMT also imparts tumour resistance to chemotherapeutic agents such as temozolomide and BCNU (*bis*-chloroethylnitrosourea).[4] This has led to the development of highly efficient inhibitors/inactivators of MGMT such as O^6-benzylguanine[5] (**2**) and O^6-(2-bromothenyl)guanine[6] (**3**) and oligodeoxyribonucleotides (ODNs) containing these modified bases display IC_{50} values in the low nM range.[7,8] However, the ability of MGMT to repair different O^6-alkGs varies considerably. For example, ODNs containing the adducts O^6-(hydroxyethyl)guanine (**4**)[8] or O^6-[4-oxo-4-(3-pyridyl)but-1-yl]guanine (**5**)[9], are repaired significantly less efficiently than those containing O^6-MeG. MGMT also repairs ODNs containing O^6-alkylguanines within DNA interstrand crosslinks (ICLs). Such ICLs can be generated following the reaction of the tricyclic N^1,O^6-ethanoguanine (**6**) (Figure 2), derived from exposure to 1,3-bis[(2-chloroethyl)-1-nitrosourea] with cytosine[10] or following the reaction of DNA with *bis* electrophiles such as busulfan (1,4-butanediol dimethanesulfonate)[11] to produce ICLs between O^6-alkG and an adjacent O^6-alkG[12] or O^4-alkylthymine.[13] The repair of these types of substrates produces covalent MGMT-DNA complexes. A synthesis of ODNs containing **6** have been described[14], whilst DNA containing the tricyclic ethanoxanthine **7** (Figure 2) is also repaired by MGMT[15] and is sufficiently stable to allow the chemical synthesis of DNA using standard phosphoramidite chemistry. ODNs containing **7** have allowed the formation of covalent MGMT-DNA complexes, enabling structural characterisation of MGMT-substrate interactions using X-ray crystallography.[3]

In common with MGMT, the highly homologous alkyltransferase-like (ATL) proteins also recognise O^6-alkG adducts in DNA.[16,17] However, since they lack a nucleophilic Cys (which is typically replaced by Trp or Ala) they bind to, but do not de-alkylate these adducts. Adduct recognition by both MGMT and ATL proteins requires flipping of the base adduct into the active site. Previous structural studies[18,19] reveal that the alkyl binding pocket in the *S.pombe* ATL protein, Atl1, is significantly larger than that in

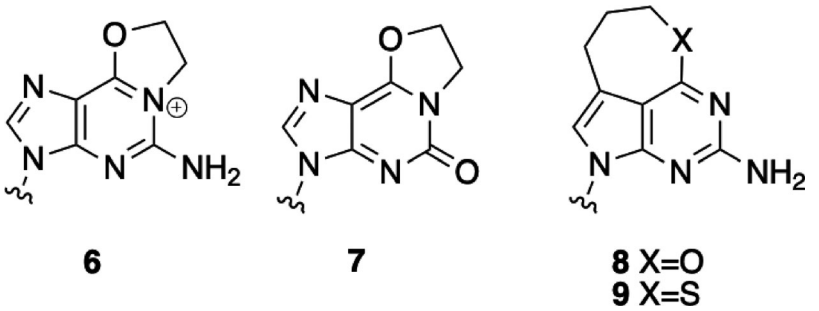


Figure 2. Tricyclic analogues of O^6-alkylguanine.

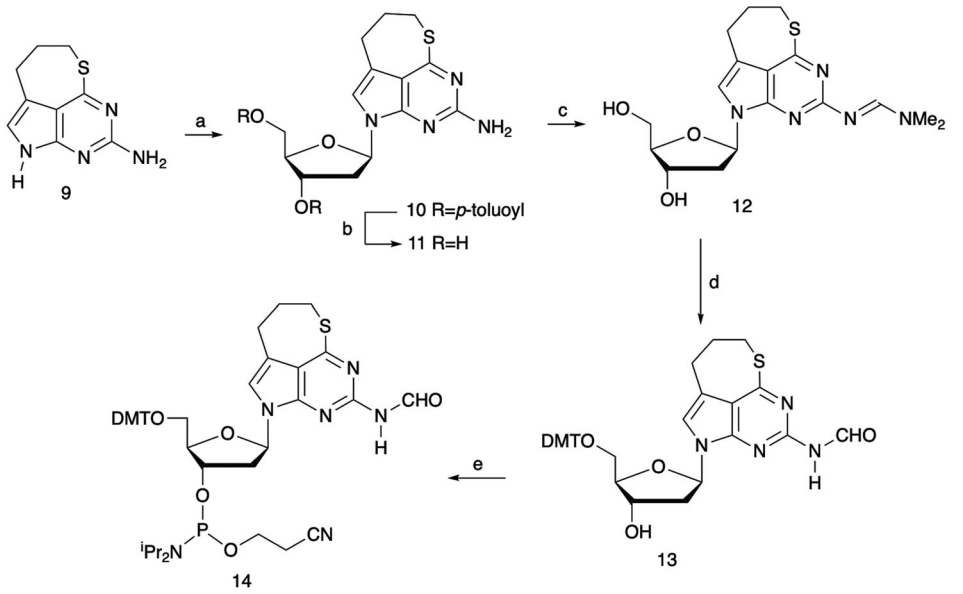
MGMT. Consequently, Atl1 can bind a much wider range of O^6-alkG adducts including many that are poor substrates for MGMT.[19]

Previously, we described[20] syntheses of the tricyclic O^6-alkyl-7-deazaguanines **8** and **9** (Figure 2), to examine key features of MGMT substrate recognition. Interestingly we found that ODNs containing compound **8** were completely refractory to repair by MGMT.[21] We reasoned that this might be due to the locked *anti* or *proximal* conformation of the alkyl group (pointing away from the hydrogen bonding face). This would place Cys145 in MGMT in an unfavourable trajectory for nucleophilic addition to the electrophilic carbon attached to the heteroatom. In contrast to **8**, the third ring of **9** is larger and likely more flexible due to the difference in the C-S vs C-O bond lengths in **9**[20] (0.4 Å longer) compared to **8**. Previous studies have revealed that ODNs containing S^6-methylthio-6-thioguanine in addition to those containing O^6-methyl-7-deazaguanine are repaired by MGMT but approximately 450 and 25 times slower respectively than O^6-MeG-containing ODNs.[22] Therefore, we were keen to explore whether this would allow DNA containing the modified base **9** to react with MGMT and form a covalent protein-DNA complex. Here we describe the synthesis of the 2′-deoxyribonucleoside of **9**, its incorporation into ODNs and the properties of these ODNs as substrates for both MGMT and Atl1.

2. Results and discussion

2.1. Synthesis of modified oligodeoxyribonucleotides

Earlier studies revealed that the heterocycle **8** was insoluble in acetonitrile[21] which is the preferred solvent for the well-established glycosylation method involving the reaction of the sodium salt of the purine or deazapurine with 1-chloro-2-deoxy-3,5-di-O-*p*-toluoyl-ß-D-ribofuranose.[23,24] Consequently the third ring of **8** was constructed after glycosylation of a suitable precursor.[21] In contrast to **8**, compound **9** was fully soluble in acetonitrile allowing direct glycosylation of the preformed heterocycle (Scheme 1). However, following silica column chromatography the



Scheme 1. Synthesis of phosphoramidite for incorporation of heterocycle **9** into ODNs. (a) NaH in CH_3CN then 1-chloro-2-deoxy-3,5-di-O-*p*-toluoyl-ß-D-ribofuranose, 3 hours, rt, (b) NaOH in aq dioxan, reflux, 1 hour, 47% from **9**, (c) $Me_2NC(OMe)_2$, DMF, rt, 12 hours, 78%, (d) DMTCl, Et_3N, DMAP, pyridine, rt, 4 hours, 68%, (e) $iPr_2NPCl(OCH_2CH_2CN)$, iPr_2EtN, CH_2Cl_2, rt, 1 hour, 60%.

protected nucleoside product (**10**) could not be obtained in pure form and contained small amounts of sugar impurities as evidenced by ^{1}H NMR. Consequently, the partially purified nucleoside **10** was deprotected by heating in 1 M aq sodium hydroxide. After silica chromatography a pure sample of the deprotected nucleoside **11** was obtained in 52% overall yield from **9**. The nucleoside **11** was then protected as the formamidine **12**, obtained in 78% yield following reaction with dimethylformamide dimethylacetal in DMF. Compound **12** was then reacted with dimethoxytrityl chloride to protect the 5′-hydroxyl group. Analysis of the product by ^{1}H NMR indicated that although the 5′-OH had been successfully protected, the amidine had decomposed to the corresponding *N*-formyl compound. Formamidine protected nucleosides are known to be relatively unstable[25] whilst we[21] and others[26] have reported on the lability of the formamidine protecting group with other O^6-alkyl-7-deazaguanine nucleosides, but have found the *N*-formyl group to function as a suitable protecting group during standard automated ODN synthesis.[21,26] The pK_a of the *N*-alkylated version of heterocycle **9** is approximately 4.8 compared to around 3.3 for deoxyguanosine and we speculate that the consequent increased basicity of the amidine **12** makes it more prone to protonation and subsequent hydrolysis. Phosphitylation of **13** provided the phosphoramidite **14** as a mixture of two diastereoisomers that were characterised by ^{31}P NMR and ESI-MS.

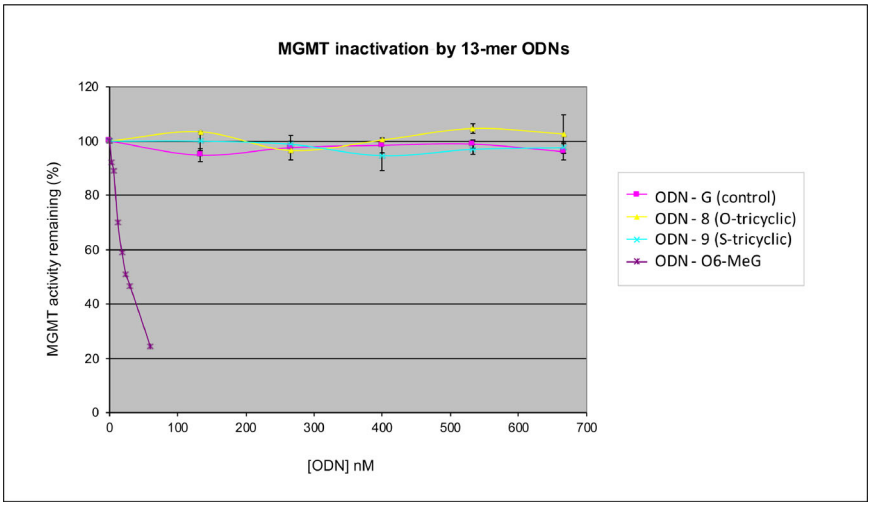


Figure 3. MGMT inactivation data with ODNs indicated.

The ODNs 5′-d(GCCATG**9**CTAGTA) bearing either a free 5′-HO (ODN-1) or a fluorescent 5′-SIMA(HEX) (dichlorodiphenylfluorescein) label (ODN-2) were then prepared using standard protocols on a DNA synthesiser but using a 0.15 M acetonitrile solution of phosphoramidite **14** (rather than a standard 0.1 M solution). "Base-labile" protecting groups were used for the other bases (phenoxyacetyl for G and A and acetyl for C). The ODNs were deprotected with 33% aq ammonia at 50 °C for 6 hours, then purified by reverse-phase HPLC and characterised by ESI-MS.

2.2. Recognition of modified oligodeoxyribonucleotides by MGMT and Atl1

MGMT repairs O^6-MeG in single stranded (ss) or duplex DNA with a similar efficiency.[27] Therefore we assessed the repair of ss ODN-1 by MGMT. For this we used the standard assay[28] that involves pre-incubation of purified recombinant protein with the ODN, followed by the addition of excess DNA containing tritium-labelled O^6-MeG. Since the MGMT reaction is irreversible the residual protein activity can be quantified by measuring the radioactivity transferred to the protein, allowing IC_{50} values to be determined. It should be noted that MGMT repairs The O^6-MeG-containing ODN exhibited an IC_{50} value of approximately 15 nM (Figure 3). In contrast, no measurable inactivation of MGMT by ODN1 was observed, in common with both the ODN containing the tricyclic oxygen-containing heterocycle **8** and control (G-containing) ODN. This indicates that MGMT is unable to repair ODNs containing either of the tricyclic compounds **8** or **9**. In addition no evidence for binding of MGMT to ODN-1 was observed following analysis by polyacrylamide gel electrophoresis (data not shown). Pertinent to compound **9** is the fact that

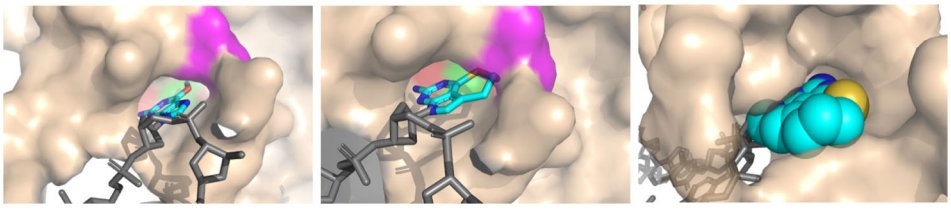


Figure 4. Left: C145S-MGMT bound to DNA containing O^6-methylguanine (PDB file 1T38 [3]); Centre: C145S-MGMT bound to DNA containing **9** (PDB accession number 1T38 [3] and CCDC297448 [20]); Right: Atl1 bound to DNA containing **9** (based on PDB file 3GX4 [18] for O^6-methylguanine-containing complex and CCDC297448 [20]). In left and centre panels, Ser145 is coloured green and Ser157 coloured pink.

Table 1. Equilibrium dissociation constants for Atl1-ODN complexes.

ODN sequence	K_D (nM)
5′-SIMA-d(GCCATG^{Me}**G**CTAGTA)	2.4 ± 1.2
5′-SIMA-d(GCCATG**9**CTAGTA)	182 ± 5
5′-SIMA-d(GCCATG**8**CTAGTA)	430 ± 41
5′-SIMA-d(GCCATG**G**CTAGTA)	740 ± 91

MGMT is able to repair ODNs containing S^6-methyl-6-thioguanine, and 7-deaza-O^6-methylguanine.[22] It therefore appears that for compound **9** the lack of repair by MGMT is due to the *anti* or *proximal* conformation of the alkyl substituent attached to the heteroatom. To gain further insight into the binding of the modified base **9** by MGMT we examined the published crystal structure of O^6-methylguanine-containing DNA bound to the catalytically-inactive MGMT-C145S mutant (Figure 4, left). When the base **9** is superimposed into this structure in place of O^6-methylguanine, a critical steric clash between the saturated third ring of the base and Ser157 arises (Figure 4, centre). These results suggest that both binding and the alkyltransfer reactions are inhibited resulting in the inability of MGMT to repair DNA containing **9**.

Next we examined the ability of Atl1 to recognise the base adduct **9** in DNA. Since Atl1 binds with very high affinity to O^6-alkylguanines in both ss and duplex DNA[18,19] we assessed its ability to recognise adduct **9** using ss ODN as previously. Thus, by titrating native, wild-type Atl1 protein into a solution containing 5′-SIMA-(HEX) labelled ODN-2. For the control O^6-MeG containing ODN, we observed a protein-dependent decrease in fluorescence from which we derived an equilibrium dissociation constant (Table 1 and Figure 5). Atl1 has a higher affinity for DNA containing **9** compared to the G-containing sequence, but in common with DNA containing the oxygen-analogue **8** [21], the affinity is dramatically decreased relative to ODNs containing O^6-MeG. Modelling analogue **9** into the crystal structure of Atl1 in complex with O^6-MeG containing DNA using PyMol (Figure 4, right) suggests that the saturated ring is in van der Waals contact with the

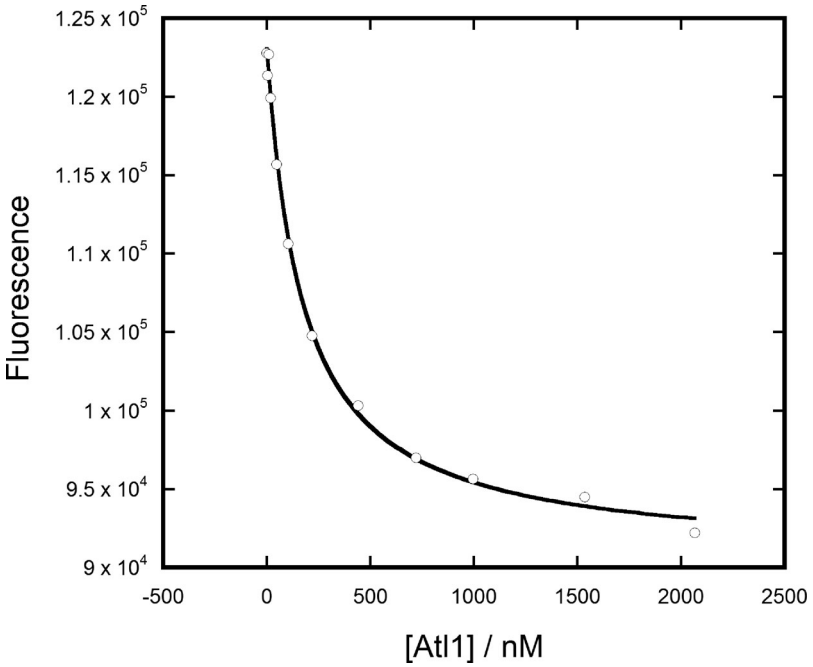


Figure 5. Fluorescence vs [Atl1] using ODN-2 (5′-SIMA-d(GCCATG**9**CTAGTA).

main chain amino acid residues in the C-terminal domain of Atl1 which affects the ability of the protein to bind the DNA.

3. Conclusions

We have prepared ODNs containing a tricyclic thio analogue of O^6-methylguanine and shown that with the alkyl group locked in the *anti* conformation blocks repair by MGMT and severely impedes the binding of Atl1. Due to the inability of MGMT to repair both compound **9** and previously described **8**[21] we anticipate that these compounds are likely to be mutagenic and highly toxic unless other mechanisms for their repair such as BER or NER are possible. We will report on these properties in due course.

4. Experimental

4.1. General methods

Dry solvents were obtained from the University of Sheffield Grubbs apparatus. 1-Chloro-2-deoxy-3,5-di-*O*-*p*-toluoyl-ß-D-ribofuranose was obtained from Carbosynth and 2-cyanoethyl-*N,N*-diisopropyl chlorophosphoramidite from SigmaAldrich. Column chromatography purifications were carried out on silica (VWR chemicals, 60–200 mesh) Thin layer chromatography

(TLC) was performed on pre-coated Merck silica gel 60 F_{254} aluminium backed plates. TLCs were visualised under UV (254 nm). NMR spectra were recorded on either a Bruker AV250, AV400 or AV500 spectrometer (individually stated for all data) and chemical shifts are reported in δ values relative to tetramethylsilane as an external standard. *J* values are given in Hz. Mass spectrometry was performed by the University of Sheffield Mass Spectrometry Service using the method of electrospray ionisation on a Waters LCT Mass Spectrometer unless otherwise stated. Analytical RP-HPLC was performed on Waters 2695 or 2690 instrumentation using a Phenomenex Gemini C18 5 µm 4.6 × 250 mm column, flow rate 1 mL/min, UV detection was recorded at 260 nm unless specified otherwise. Preparative RP- HPLC performed using a Phenomenex Gemini C18 5 µm 110 Å 21.2 × 250 mm column at a flow rate of 21 mL/min. UV detection was recorded at 260 nm unless specified otherwise. All quoted retention times are those found by analytical HPLC.

4.2. Chemical synthesis

NMR Spectra for the compounds synthesised can be found in supplementary information.

4.2.1. 4-Amino-2-[2-deoxy-3,5-di-O-(p-toluoyl)-β-D-erythro-pentofuranosyl]-6-thia-7,8,9-trihydro-2,3,5-triazabenzo[cd]azulene (10)

Compound **9** [20] (206 mg, 1.0 mmol) was dissolved in anhydrous MeCN (15 mL) under Ar at room temp. NaH (44 mg, 1.1 mmol, 60% in mineral oil) was added, the mixture stirred for 1 hour then 1-chloro-2-deoxy-3,5-di-*O*-*p*-toluoyl-ß-D-ribofuranose (602 mg, 1.4 mmol) was added portionwise over 10 minutes. After stirring for 3 hours, the solvent was evaporated and the product purified by silica column chromatography (eluent 7% EtOAc in DCM) to give a pale yellow foam (500 mg). The nucleoside could not be obtained free from sugar impurities and was further deprotected (to **11**) as described below.$\boldsymbol{R_f}$ (20% ethyl acetate in DCM) 0.5 (fluorescent at 356 nm). **^{1}H NMR** (250.13 MHz, $CDCl_3$) δ_H 8.02-7.95 (m, 4H), 7.32-7.26 (m, 4H), 6.74 (s, 1H), 6.68 (dd, 1H, $J = 3.4$ Hz, 5.5 Hz), 5.76-5.72 (m, 1H), 5.02 (s, 2H), 4.78-4.72 (m, 1H), 4.62-4.55 (m, 2H), 3.12-3.09 (m, 2H), 2.84-2.76 (m, 1H), 2.67-2.60 (m, 1H), 2.46 (s, 3H), 2.45 (s, 3H), 2.30-2.27 (m, 2H) ppm **^{13}C NMR** (100.62 MHz, $CDCl_3$) δ_C 166.26, 166.06, 163.30, 157.45, 151.67, 144.40, 144.07, 129.83, 129.70, 129.25, 126.98, 126.65, 117.82, 115.71, 110.27, 83.11, 81.83, 75.27, 64.30, 39.35, 31.03, 29.54, 28.55, 21.70 ppm **HR ES-MS** m/z calcd for $C_{30}H_{31}N_4O_5S$ $[M+H]^+$ 559.2015, found 559.2021.

4.2.2. 4-Amino-2-(2-deoxy-β-D-erythro-pentofuranosyl)-6-thia-7,8,9-trihydro-2,3,5-triazabenzo[cd]azulene (11)

Compound **10** (500 mg, 895 µmol) was dissolved in 1,4-dioxane (6 mL) and 1 M aq NaOH solution was added (3 mL, 3 mmol). The mixture was then refluxed at 90 °C for 1 hour, cooled to r.t. and neutralised with 0.1 M acetic acid aqueous solution. The mixture was evaporated, and the residue purified by silica column chromatography (eluent 60% acetone in DCM) to obtain a pale yellow solid (150 mg, 466 µmol, 47% from **9**). $\boldsymbol{R_f}$ (20% MeOH in DCM) 0.28 (fluorescent spot using 356 nm) **^{1}H NMR** (500.13 MHz, d6-DMSO) δ_H 7.00 (s, 1H), 6.42 (dd, 1H, $J = 3.4$ Hz, 5.5 Hz), 6.16 (s, 2H), 5.19 (d, 1H, $J = 4.5$ Hz), 4.87 (t, 1H, $J = 5.6$ Hz), 4.29-4.25 (m, 1H), 3.75-3.73 (m, 1H), 3.51-3.43 (m, 2H), 3.07 (t, 2H, $J = 6.0$ Hz), 2.85 (t, 2H, $J = 7.6$ Hz), 2.36-2.30 (m, 1H), 2.13 (m, 2H), 2.07-2.02 (m, 1H) **^{13}C NMR** (125.76 MHz, d6-DMSO) δ_C 165.16, 158.13, 152.55, 117.76, 117.76, 114.10, 109.41, 86.82, 81.47, 71.02, 62.06, 39.84, 31.41, 29.96, 29.03; **HR ES-MS** m/z calcd for $C_{14}H_{19}N_4O_3S$ $[M+H]^+$ 323.1178, observed 323.1183.

4.2.3. 4-{[(Dimethylamino)methylidene]amino}-2-(2-deoxy-β-D-erythro-pentofuranosyl)-6-thia-7,8,9-trihydro-2,3,5-triazabenzo[cd]azulene (12)

Compound **11** (140 mg, 435 µmol) was dissolved in anhydrous DMF (2 mL), under Ar at room temperature. After 1 hour, *N,N*-dimethylformamide dimethylacetal (0.5 mL, 3.5 mmol) was added and the solution left to stir overnight. After evaporation the residue was purified by silica column chromatography (eluent 0-5% MeOH in DCM (containing 1% triethylamine)) to give a white foam (128 mg, 78%). $\boldsymbol{R_f}$ (20% MeOH in DCM) 0.35 **^{1}H NMR** (249.87 MHz, CD_3OD) δ_H 8.63 (s, 1H), 7.20 (s, 1H), 6.67 (dd, 1H, $J = 8.0$ Hz, 6.1 Hz), 4.49 (m, 1H), 3.95 (m, 1H), 3.73 (m, 2H), 3.25 (m, 1H), 3.18 (s, 3H), 3.16 (m, 2H), 3.12 (s, 3H), 2.98 (m, 2H), 2.60 (m,1H), 2.34 (m, 3H). **^{13}C NMR** (100.62 MHz, CD_3OD) δ_C 164.49, 158.44, 156.66, 150.30, 118.99, 113.23, 111.55, 85.49, 81.53, 69.98, 60.71, 38.40, 38.03, 32.55, 30.39, 28.54, 27.35. **HR ES-MS** m/z calcd for $C_{17}H_{24}N_5O_3S$ $[M+H]^+$ 378.1600, observed 378.1603

4.2.4. 4-{[(Formylamino}-2-(2-deoxy-5-O-[4,4′-dimethoxytrityl]-β-D-erythro-pentofuranosyl)-6-thia-7,8,9-trihydro-2,3,5-triazabenzo[cd]azulene (13)

Compound **12** (100 mg, 265 µmol) was dissolved in anhydrous pyridine (5 mL) under Ar with stirring at room temperature. Triethylamine (0.5 mL, 340 µmol) , 4,4-dimethoxytrityl chloride (109 mg, 318 µmol) and 4-*N,N*-dimethylaminopyridine (2 mg, 15 µmol) were added. After 4 hours the mixture was evaporated, redissolved in EtOAc (20 mL) and washed with water (10 mL), brine (10 mL) and dried (Na_2SO_4). Evaporation and purification

of the resulting residue by silica column chromatography (0-5% MeOH/DCM (1% Et_3N)) gave **13** as a pale yellow foam (117 mg, 68%). $\boldsymbol{R_f}$ (10% MeOH in DCM) 0.35 **^{1}H NMR** δ (249.87 MHz, CD_3OD) δ_H 9.41 (s, 1H), 8.54 (d, 1H), 7.43–7.15 (m, 14H), 6.65 (t, 1H, $J = 6.6$ Hz), 4.64 (m, 1H), 4.05 (m, 1H), 3.77 (s, 6H), 3.32 (m, 3H), 3.12 (m, 2H), 2.75–2.64 (m, 3H), 2.41 (m, 1H), 2.18 (m, 2H). **^{13}C NMR** (100.62 MHz, CD_3OD) δ_C 166.54, 163.17, 158.50, 150.64, 150.27, 149.13, 144.67, 135.80, 135.69, 130.17, 130.13, 128.25, 127.79, 120.85, 115.01, 114.57, 113.06, 106.57, 86.45, 86.08, 82.94, 71.54, 63.98, 55.17, 46.16, 46.07, 40.80, 38.99, 32.47, 29.88, 29.12, 11.35. **HR ES-MS** (ESI) m/z calcd for $C_{36}H_{37}N_4O_6S$ $[M+H]^+$ 653.2434, observed 653.2458

4.2.5. 4-[(Formyl)amino)-2-[2-deoxy-3-(2-cyanoethyl-N,N-diisopropylaminophosphoryl-5-O-(4,4′-dimethoxytrityl))-β-D-erythro-pentofuranosyl)]-6-thia-7,8,9-trihydro-2,3,5-triazabenzo[cd]azulene (14)

Compound **17** (110 mg, 168 µmol) was dissolved in dry DCM (2 mL) under Ar at room temperature. Dry *N,N*-diisopropyl ethylamine (116 µL, 672 µmol) was then added followed by the dropwise addition of 2-cyanoethyl-N,N-diisopropyl chlorophosphoramidite (48 µL, 200 µmol) over 15 minutes. After 1 hour benzyl alcohol solid support (2.5 mmol/g average) was added[29] and the resulting suspension was stirred for 30 minutes further. The reaction was then filtered to remove the solid support, the filtrate was washed with 10% Na_2CO_3 (10 ml), brine (10 mL) and dried (Na_2SO_4). The residue was evaporated and purified by silica gel column chromatography under nitrogen eluting with 45% dichloromethane/hexane (containing 10% triethylamine) to give phosphoramidite **14** as a white foam (85 mg, 60%). $\boldsymbol{R_f}$ (EtOAc/hexane/Et_3N, 45:45:10 = 0.55, 0.57; **^{31}P NMR** ($CDCl_3$) δ_P +148.84, +148.78 ppm; **^{1}H NMR** (249.87 MHz, $CDCl_3$) δ_H 9.48 (d, 1H), 7.80 (m, 1H). 7.46-6.79 (m, 14H), 6.65 (t, 1H, $J = 7$ Hz), 4.74 (m, 1H), 4.23 (m, 1H), 3.78 (s, 6H), 3.75 (m, 4H), 3.30 (m, 2H), 3.12 (m, 2H), 2.76 (m, 2H), 2.64 (m, 3H), 2.26 (m, 2H), 2.02 (bs,2H), 1.22 (d, 12H) **HR ES-MS** m/z calcd for $C_{45}H_{54}N_6O_7PS$ $[M+H]^+$ 853.3512, observed 853.3486

4.3. Oligodeoxyribonucleotide (ODN) synthesis

The ODNs 5′-d(GCCATG**9**CTAGTA) bearing either a 5′-OH (ODN-1) or a 5′- dichlorodiphenylfluorescein (SIMA(HEX)) label (ODN-2) were synthesised on an Applied Biosystems DNA automated synthesiser (Model 394) employing "base labile" phosphoramidites (pac-A, iPr-pac-G and Ac-C) from Link Technologies and the phosphoramidite for the 5′-SIMA label from Glen Research. The coupling efficiency for the phosphoramidite **14** (based on trityl cation measurement) was 98%. After synthesis, the ODNs

were removed from the solid support with 33% aq ammonia solution at room temperature by the Applied Biosystems 394 automated synthesiser through the machine's own deprotection programme. Further incubation at room temperature overnight removed the *N*-formyl and other protecting groups. The deprotected oligomers were purified by reverse phase high performance liquid chromatography (RP-HPLC) using a Hichrom ACE-5 C18 (250 × 4.6 mm) column with a flow rate of 1 mL/minute and detection at 260 nm and a gradient of 0 - 60% B in 30 minutes (A = 0.1 M triethylammonium bicarbonate pH 7.5/5% CH_3CN, B = CH_3CN). Fractions containing purified ODNs were evaporated, redissolved in water, de-salted using a NAP-10 gel filtration column (GE Healthcare) to give 286 nmol of ODN-1 and 231 nmol of ODN-2. ODNs were characterised using ES-MS. ODN-1 had an HPLC retention time of 14.9 minutes and ES-MS-ve calcd. 4030, found. 4030 $[M\text{-}H]^-$. ODN-2 HPLC had a retention time of 18.2 minutes and ES-MS -ve calcd. 4788, found 4788 $[M\text{-}H]^-$.

4.4. MGMT assays

The inactivation of recombinant human MGMT by the modified ODN-1 involved pre-incubation of MBP-MGMT (MGMT fusion with maltose binding protein) with varying amounts of ODN for 10 hours at 37 °C using the procedure described previously.[28]

4.5. Binding assays with Atl1

Fluorescence emission intensity measurements (determined in triplicate) used a 1 nM solutions of 5′-ODNs in 1 mL of titration buffer (50 mM Tris–HCl pH 7.5, 50 mM NaCl, 1 mM EDTA) to which were added native Atl1.[19] The binding isotherms were fitted by non-linear least-squares regression using KaleidaGraph to the following equation describing the equilibrium D + E ↔ DE (where D = ODN, E = enzyme, DE = ODN-enzyme complex)

$I = I_{max} + [(D + E + K_D) - ((D + E + K_D)^2 - (4DE))^{0.5}] (I_{min} - I_{max})/2D$ (where I = intensity measured at a certain concentration of enzyme, I_{max} = maximum intensity (i.e., prior to protein addition), I_{min} = minimum intensity (i.e., when binding is saturated), D = ODN concentration, K_D = dissociation constant).

Conflict of interest

The authors declare no conflict of interest

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